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Molecular Analysis of the 5α-Steroid Reductase Type 2 Gene in a Family With Deficiency of the Enzyme

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This report describes the identification of a point mutation in the 5α-reductase type 2 (5α-SR2) gene from a family in which both sibs (6 and 3 years old) have steroid 5α-reductase 2 deficiency. The five exons of the gene were individually amplified by the polymerase chain reaction (PCR) and analysed for single-strand conformation polymorphisms (SSCP) to detect mutations. Direct sequencing of the mutant PCR products demonstrated a single C→T mutation, within exon 4, changing codon 227 from CGA (Arg) to TGA (premature termination signal). Both patients were homozygous for the mutation, but their parents were heterozygous. These results suggest that the mutation at codon 227 impairs normal 5α-SR2 function, thus leading to the phenotypical expression of this rare enzymatic defect. Am. J. Med. Genet. 69:69–72, 1997.

KEY WORDS: steroid 5α-reductase; 5α-reductase type 2; steroid 5α-reductase 2 deficiency; male pseudohermaphroditism

INTRODUCTION

Steroid 5α-reductase 2 deficiency (5α-SR2D) is an autosomal recessive enzymatic disorder, which is expressed as a specific form of male pseudohermaphroditism. In this entity, the conversion of testosterone (T) to 5α-dihydrotestosterone (DHT) is impaired. Affected individuals are 46,XY males who, at birth, generally present ambiguous external genitalia characterized by perineoscrotal hypospadias with pseudovagina, microphallus, cryptorchidism [Imperato-McGinley et al., 1974; Walsh et al., 1974; Wilson et al., 1993]. Due to their phenotypic appearence, most of these patients are reared as females. During puberty virilization occurs, and without any therapeutic maneuver, masculinization is frequently accompanied by a gender identity change from female to male [Pérez-Palacios et al., 1987; Méndez et al., 1995]. The gene responsible for this deficiency was isolated and its intron-exon boundaries were determined recently [Andersson et al., 1991; Labrie et al., 1992]. The complete nucleotide sequence of the type 2 steroid reductase (5α-SR2) gene shows that its structural organization consists of 5 exons and 4 introns. In humans, it is located on chromosome 2 (p23 region) and encodes for a protein of 254 amino acids [Andersson et al., 1991; Labrie et al., 1992]. This enzyme, which catalyzes the reduction of T to DHT [Labrie et al., 1992], has an optimal pH of 5.5 [Wilson et al., 1993] and is expressed predominantly in androgen-dependent tissues [Silver et al., 1992]. Results from molecular studies have demonstrated that mutations in the *5α-SR2* gene account for this disorder [Thigpen et al., 1992; Wigley et al., 1994]. To date, more than 25 different point mutations, as well as a splice-junction alteration and two nonsense codons have been reported [Thigpen et al., 1992; Wilson et al., 1993]. Each of the substitution mutations alters an amino acid that is conserved among the sequenced rat and monkey 5α-reductases [Normington et al., 1992; Levy et al., 1995].

CLINICAL REPORT

We report on two sibs with 5α-SR2 deficiency. The type 2 gene of each relative was screened for mutations using polymerase chain reaction (PCR) amplifications and single-strand conformation polymorphism (SSCP) analysis. The propositi were two sibs with a female gender identity, 6 (patient 1) and 3 (patient 2) years old, who were refered because of genital ambiguity. There was no known consanguinity, nor family history of the disease; however, both parents were born in the same

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TABLE I. Oligonucleotides Used for Amplification of Each Exon of the 5α-SR2 Gene

Amplification target	Primer name	Sequence (5′ → 3′)[a]	PCR fragments length (bp)	Annealing temperature (°C)
Exon 1	501	GCAGCGGCCACCGGCGAGG	358	65
	502	AGCAGGGCAGTGCGCTGCACT		
Exon 2	503	TGAATCCTAACCTTTCCTCCC	235	58
	504	AGCTGGGAAGTAGGTGAGAA		
Exon 3	505	TGTGAAAAAAGCACCACAATCT	208	58
	506	GAGGGAAGAGTGAGAGTCTGG		
Exon 4	507	TGATTGACCTTCCGATTCTT	232	54
	508	TGGAGAAGAAGAAAGCTACGT		
Exon 5	509	TCAGCCACTGCTCCATTATAT	166	58
	510	CAGTTTTCATCAGCATTGTGG		

[a] Designed from the 5α-SR2 sequence reported by Labrie et al. [1992].

village. Their ethnic origin was Mexican-Mestizo. Both patients had perineoscrotal hypospadias with pseudovagina, a short phallus (1.7 and 1.5 cm in length) and bilateral testes, which were palpable within the labioscrotal folds. Their karyotype was 46,XY, the basal T/DHT ratio was 2 in both cases; however, it increased to 38 and 36 after an hCG stimulation test (1,500 IU/4 days).

METHODS

Genomic DNA for PCR was isolated from blood leucocytes of the patients, their parents and controls (unrelated normal individuals) by standard methods. Five sets of oligonucleotide primers (Table I) were used in the amplification of exons 1 to 5 (including their flanking intronic sequences) of the 5α-SR2 gene. Reactions were denatured at 94°C for 3 minutes and subjected to 25 cycles of amplification at 94°C for 1 minute, annealing at 54–65°C (see Table I) for 1 minute and extension at 72°C for 1 minute. After amplification, PCR products were analyzed in ethidium bromide-stained agarose gels and found to be single bands of the predicted size. Subsequently, each of the exons was purified on Centricon-100 columns (Amicon Inc.) and used as template in SSCP analysis and dideoxy chain-termination reactions [Sanger et al., 1977]. SSCP analysis of PCR products was performed according to the method of Orita et al. [1989], using [α-^{32}P] dCTP [Thigpen et al., 1992]. Direct sequencing of the PCR fragments was carried out with the AmpliCycle sequencing kit (Perkin Elmer-Roche, Branchburg, NJ) using ^{32}P end-labelled primers. Fibroblasts established from genital skin biopsies of patients 1 and 2 and normal unrelated subjects were maintained in Minimum Essential Medium (MEM, Gibco-BRL), containing 10% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Control and patient genital skin fibroblasts were grown to confluence at 37°C in 5% CO_2. 5α-reductase activity was measured at pH 5.5 in extracts of fibroblasts (sonicates), and at pH 7.4 in whole cells (monolayers) as previously described [Méndez et al., 1995].

RESULTS

In all cases five exons were amplified to the expected sizes by PCR, indicating the absence of gross deletions or rearrangements within these fragments. By SSCP analysis no abnormalities were detected in the migration patterns of single-stranded DNAs derived from exons 1, 2, 3, and 5 of the family (data not shown). However, PCR products from exon 4 of the affected sibs (P_1,P_2) displayed altered electrophoretic mobility as compared with that of the controls (Fig. 1). Exon 4 DNA of the parents (M,F), exhibited the mutant band (with "fast" mobility) and the normal wild-type banding pattern (Fig. 1). Sequencing of the amplified exon 4 from the patients (Fig. 2), showed a C→T mutation at nucleotide 679, changing codon 227 from CGA (arginine) to TGA (***premature termination signal); both were homozygous for the mutant Arg 227*** allele. Likewise, sequence analysis confirmed that the parents were heterozygous for the same mutation, both having

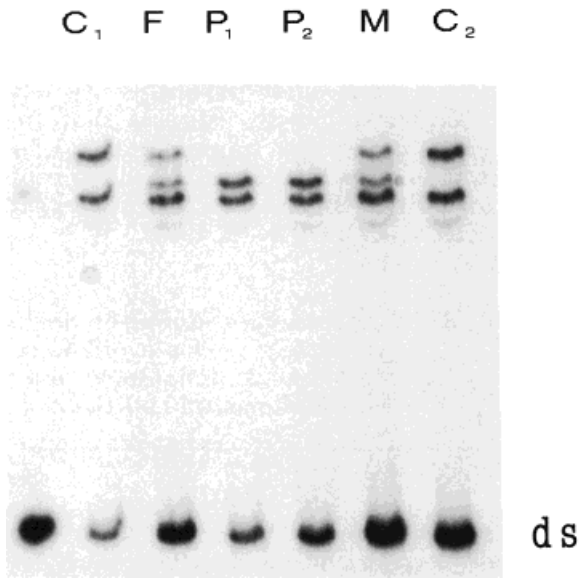

Fig. 1. Single-strand conformational polymorphism analysis of exon 4 of the 5α-reductase 2 gene. Exon 4 and its flanking intronic sequences were amplified using genomic DNA from two prepubertal subjects (P_1, P_2) with 5α-reductase deficiency and their father (F) and mother (M). Two unrelated normal subjects (C_1 = male; C_2 = female) served as controls. After denaturation, labelled PCR products were electrophoresed on a neutral 8% polyacrylamide gel containing 10% glycerol. After electrophoresis the gel was dried and subjected to autoradiography. The mobility shift of one of the strands in P_1 and P_2 denotes the presence of a mutant allele. Heterozygosity in the parents (F,M) is pointed out by three bands indicating the presence of both normal and mutant alleles. The migration pattern of nondenatured double strand DNA is indicated (ds).

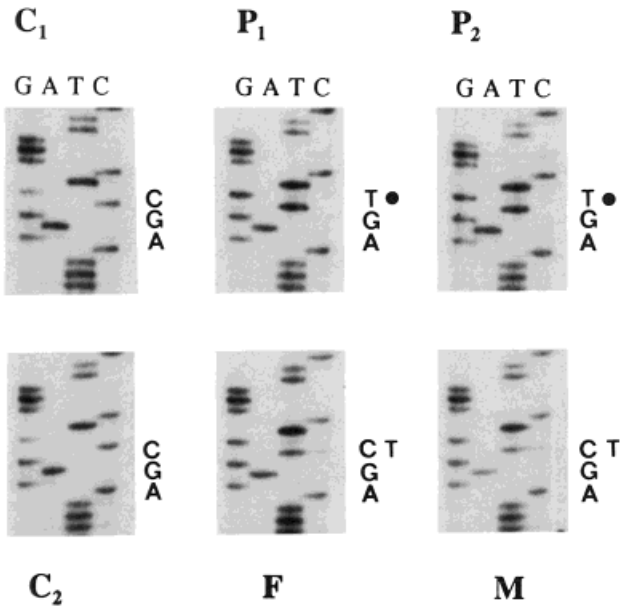

Fig. 2. Partial nucleotide sequence of exon 4 of the steroid 5α-reductase 2 gene showing a single (C→T) base sustitution in 2 sibs with 5α-reductase deficiency. The affected individuals (P_1 and P_2) are homozygous for a stop codon mutation at codon 227 changing Arg (CGA) to a TGA. Their parents (F,M) are heterozygous (CGA/TGA) for the mutation. Exon 4 DNA from two unrelated normal subjets (C_1 and C_2) served as reference.

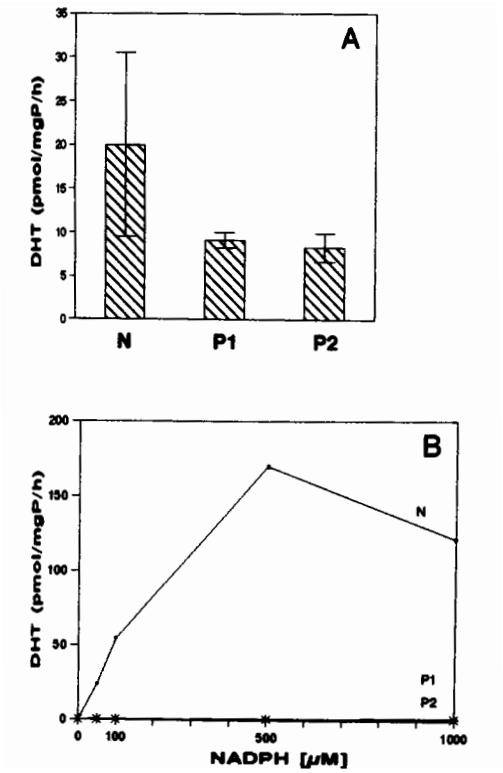

Fig. 3. 5α-reductase activity in fibroblasts cultured from genital skin biopsies of patients with 5α-reductase deficiency (P_1, P_2) and normal subjets (N). Enzyme activity was assessed in vitro through the conversion of [^{3}H] testosterone to 5α-dihydrotestosterone (DHT). Assays were performed at pH 7.4 using whole cells in monolayer (**A**) and at pH 5.5 in cellular sonicates containing increasing concentrations of NADPH (**B**). In panel A, each bar shows mean values ± SD from 6 (N) or 3 (P_1, P_2) independent determinations assayed in duplicate.

the normal (CGA) and the mutant (TGA) allele (Fig. 2). Sequencing analysis of exon 1 showed that both patients were homozygous for valine (GTA) at codon 89 whereas their parents exhibited an heterozygous pattern (GTA) Val/(CTA) Leu, at the same position (data not shown). In in vitro assays, the cultured fibroblasts of these patients exhibited a decreased enzymatic activity; under the same experimental conditions, the conversion of [^{3}H]T to DHT was consistently lower in the mutant cells than in normal controls (Fig. 3).

DISCUSSION

In this study we have identified a single base mutation in the 5α-reductase type 2 gene of two prepubertal sibs bearing 5α-SR2D. Both patients had the homozygous mutation of codon 227 (CGA) to a stop codon TGA. The molecular lesion identified might lead to the formation of nonfunctional enzyme resulting in this disorder. Studies of site-directed mutagenesis have shown that almost all mutations in the 5α-SR2 gene impair enzyme function by affecting either substrate or cofactor binding [Wigley et al., 1994]. The mutation of codon 227 is located within the region considered as the functional domain for NADPH binding [Wilson et al., 1993]. Although this mutation has not been so far recreated and/or expressed in transfection studies, the results from biochemical assays allow to speculate that this stop codon mutation may impair the enzyme affinity for its cofactor. Figure 3 shows the formation of DHT from [^{3}H]T in cultured fibroblasts from genital skin of these patients. Under the experimental conditions employed, the 5α-reductase activity in mutant cells was undetectable at pH 5.5, whereas in normal cells it increased alongside increased cofactor concentrations (Fig. 3). Thus, arginine 227 appears essential for normal 5α-SR2 function. Apart from C→T mutation in exon 4, no other abnormalities within the coding region of the gene were detected. However sequencing analysis of exon 1 showed homozygocity in both patients at codon 89 and heterozygocity in both parents. Such variation could indicate a genetic polymorphism, since both conditions (Val 89 or Leu 89) were originally described for the wild type gene [Andersson et al., 1991; Labrie et al., 1992]. The C→T transition reported herein represents the third nonsense mutation described in the 5α-SR type 2 gene. It was previously found in 2 unrelated individuals, one of them of Mexican origin [Thigpen et al., 1992]. Interestingly, in all cases the mutation occurs within exon 4 at the same nucleotide (679), suggesting that this particular site is a mutational "hot spot" in the gene. The results from this study allowed us to conclude that the cause of these patients with classical form of 5α-SR deficiency was the genetic deffect of the type 2 5α-SR gene.

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